# Forum Review

# Not Every Disulfide Lasts Forever: Disulfide Bond Formation as a Redox Switch

KATRIN LINKE and URSULA JAKOB

## ABSTRACT

**Cellular compartments differ dramatically in their redox potentials. This translates directly into variations in the extent of disulfide bond formation within proteins, depending on their cellular localization. It has long been assumed that proteins that are present in the reducing environment of the cytosol do not possess disulfide bonds. The recent discovery of a number of cytosolic proteins that use specific and reversible disulfide bond formation as a functional switch suggests that this view needs to be revised. Oxidative stress-induced disulfide bond formation appears to be the main strategy to adjust the protein activity of the oxidative stress transcription factors Yap1 and OxyR, the molecular chaperone Hsp33, and the anti-sigma factor RsrA. This elegant and rapid regulation allows the cells to respond quickly to environmental changes that manifest themselves in the accumulation of reactive oxygen species.** *Antioxid. Redox Signal.* 5, 425–434.

## TO LIVE WITH OXYGEN IS TO LIVE WITH RADICALS

**R**EACTIVE OXYGEN SPECIES (ROS) are produced in cells as an unavoidable consequence of aerobic life. They arise during normal metabolism as toxic by-products of respiration or other oxidation reactions. ROS such as hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($\cdot OH$), superoxide anions ($O_2^-$) and nitric oxide (NO) are known to cause damage to proteins, nucleic acids, lipids, and other macromolecules (for review, see 62). Superoxide anions, for example, oxidatively destroy iron-sulfur clusters in proteins. This causes the release of iron, which in turn cleaves $H_2O_2$ via the Fenton reaction to produce the even more reactive hydroxyl radicals. These then damage most biomolecules directly and in proteins cause side-chain oxidation, disulfide bond formation, and fragmentation (5).

To counteract this problem, all aerobically growing organisms express a set of proteins and molecules that eliminate ROS, including catalases, peroxidases, and superoxide dismutases. In addition, there are a number of proteins and small molecules that maintain the reducing environment of the cytosol (2). In *E. coli*, two semiindependent thiol-redox pathways exist, the thioredoxin and the glutathione system (for recent reviews, see 49, 56). The thioredoxin system consists of the two small thioredoxins Trx1 (*trxA*) and Trx2 (*trxC*) that reduce disulfide bonds in cytosolic proteins by direct thiol–disulfide exchange reactions. The reducing character of the two thioredoxins is restored by the action of thioredoxin reductase (TrxB), which itself draws electrons from NADPH. The glutaredoxin system, on the other hand, consists of the small reducing tripeptide glutathione (GSH), glutathione oxidoreductase (GOR), and three glutaredoxins (Grx1–3). Reduced and oxidized glutathione (GSH and GSSG) constitute the main redox buffer in the cytosol of *E. coli* and many other organisms. In *E. coli*, the intracellular concentration of glutathione is ~5 m$M$ and the GSH to GSSG ratio is approximately 200:1 (3). This equilibrium, which translates into a redox potential of $-260$ to $-280$ mV under nonstress conditions (20), is maintained by glutathione oxidoreductase. GSH is responsible for reducing disulfide bonds in cytosolic proteins. It does this by attacking disulfide bonds and forming

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109–1048.

mixed glutathione-disulfide adducts. Glutaredoxins then resolve these mixed glutathione-disulfide bonds, maintaining the reducing character of the cytosol. The main task of these systems is therefore to prevent disulfide stress, *i.e.,* the accumulation of unwanted disulfide-bonded cytosolic proteins (2).

When ROS concentrations rise above a certain level, these scavengers are no longer sufficient to prevent oxidative damage, putting the cell into a state that is defined as oxidative stress (59). This appears to be a major factor in aging (16), and has been implicated in numerous diseases such as Alzheimer's disease, diabetes, and cancer (1, 37). It is also part of a strategy that is used by phagocytes to defend against pathogenic bacteria. Upon engulfment of the microorganism, macrophages release high concentrations of superoxides that dismute to $H_2O_2$ and hydroxyl radicals that kill the invading microorganisms (47).

Similar to other stress situations like heat shock or amino acid starvation, oxidative stress induces a highly conserved response in both procaryotes (74) and eucaryotes (19, 45). This antioxidant defense includes increased expression of molecules that eliminate ROS and maintain the redox balancing system of the cell, as well as chaperones, proteases, and DNA repair enzymes to fix the oxidative damage that occurs to proteins and DNA. In *E. coli*, two transcription factors are responsible for the induction of antioxidant gene products: OxyR, which is activated by $H_2O_2$, and SoxR, which is activated by superoxide anions (for recent reviews, see 34, 53). OxyR and SoxR are prototypes for a rapidly expanding group of redox-regulated proteins. These proteins are not damaged by ROS, but rather use ROS and/or the redox potential of the environment to quickly and reversibly change their protein activities. The mechanisms by which redox-regulated proteins sense differences in their redox environment are becoming increasingly diverse and range from reversible oxidation of Fe-S centers (53, 58), and oxidative modification of single cysteines (18, 28) to the reversible formation of intra- or intermolecular disulfide bonds (30, 73).

This review focuses on a subset of redox-regulated proteins that use highly reactive cysteines to sense changes in the cellular redox potential and that undergo reversible disulfide bond formation to alter their protein conformation and, most importantly, their activity. These proteins that use reversible disulfide bond formation as their redox switch need to be distinguished from redox-active enzymes such as thioredoxins and glutaredoxins. These enzymes work as oxidoreductases and use thiol–disulfide exchange reactions in their catalytic cycle to interact with other proteins and to transfer redox equivalents.

## DISULFIDES—MANY WAYS TO MAKE A BOND

For many years, studies of disulfide bonds focused on their role in protein folding processes and in enhancing the structural stability of exported proteins (13, 69). This view changed when reversible disulfide bond formation was identified as part of the catalytic reaction mechanism in oxidore-

ductases (20), as a mechanism for transporting reducing equivalents (6, 12), and as an important regulator of transcriptional and posttranslational processes (53).

The formation of disulfide bonds in proteins is dependent on the redox potential of the surrounding environment and, thus, their distribution within the cell is quite uneven. Disulfide bond formation is rather rare within the reducing environment of the cytosol. In contrast, oxidation of cysteines to disulfide bonds is favored in compartments with an oxidizing environment, such as the periplasm in procaryotes or the endoplasmic reticulum in eucaryotic cells (13). Here, the presence of oxidoreductases such as protein disulfide isomerase and Ero1p in the endoplasmic reticulum or DsbA and DsbB in the periplasm allows the formation of disulfide bonds in translocated proteins (12, 17).

Structural disulfide bonds in translocated proteins contribute to their protein stability. Disulfide bonds that are formed within cytosolic proteins under conditions of oxidative stress, however, are often nonspecific and can cause irreversible damage to the proteins (5). The formation of these bonds is caused either indirectly via changes in the redox status of the cytoplasm (61) or directly by ROS such as $H_2O_2$ or hydroxyl radicals. Stewart *et al.* showed that a defect in thioredoxin reductase (TrxB) results in the accumulation of disulfide bonds in cytosolic proteins, a phenomenon that is dependent on the presence of thioredoxin (61). This suggested that in the absence of the thioredoxin reductase TrxB, both thioredoxins (Trx1 and Trx2) accumulate in their oxidized form and become protein oxidants (30, 61). This protein-based introduction of disulfide bonds presumably follows typical thiol–disulfide exchange reactions, where a nucleophilic attack of a thiolate anion ($S^-$) of a protein (P) occurs on the disulfide bond of the thioredoxins (Trx) to generate a mixed disulfide bond (see Scheme 1). This mixed disulfide bond is then resolved by an intramolecular attack of a second thiolate anion, a reaction that leads to the oxidation of the protein and the reduction of thioredoxin (12).

Accumulating ROS like $H_2O_2$ can directly oxidize protein thiols *in vitro* and *in vivo* (5). $H_2O_2$ oxidizes thiol groups to sulfenic acid (R-SOH), which is rather unstable and reactive. It rapidly forms stable intra- or intermolecular disulfide bonds if other thiol groups are present in the vicinity (9). However, in the absence of proximal thiol groups and when located in a suitable structural microenvironment, sulfenic acid can be stable. This formation of a stable sulfenic acid is, for instance, the basis of a different type of redox regulation in proteins such as Fos and Jun kinases (for review, see 9). Alternatively, interaction of $H_2O_2$ with trace amounts of transition metals such as Fe(II) or Cu(I) can lead to the formation of hydroxyl radicals via the Fenton reaction (see Scheme 2) (64). These hydroxyl radicals then attack thiol groups and form the even more reactive thiyl radicals that quickly form intra- and intermolecular disulfide bonds with other thiol groups that are
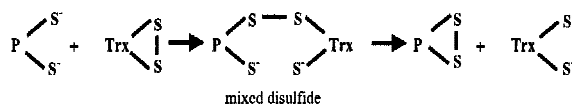


**Scheme 1.**
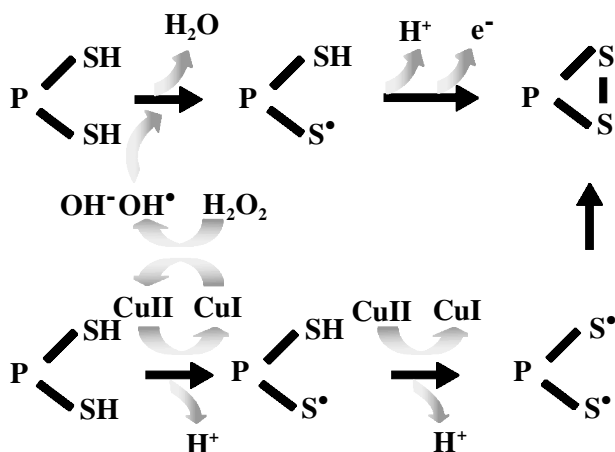
**Scheme 2.**

present in the vicinity. What feeds this vicious cycle is the direct reaction between oxidized Cu(II) and Fe(III) with thiols, a reaction that regenerates the Fenton reagents while forming more thiyl radicals on cysteines (see Scheme 2) (70).

While nonspecific and nonnative disulfide bonds are accumulating in cytosolic proteins, specific and native disulfide bonds are being formed in redox-regulated proteins such as the transcription factor OxyR and the molecular chaperone Hsp33. This activates the transcription factor OxyR, which induces the expression of antioxidant genes in procaryotes (73) and turns on Hsp33's chaperone activity, which protects cytosolic proteins against oxidative stress damage (30).

## SWITCHING ON OXYR: MAKING CONTACT WITH RNA POLYMERASE

OxyR is a LysR-type transcription factor that is responsible for the regulation of the antioxidant defense in procaryotic organisms (8). Upon exposure of cells to $H_2O_2$, nitrosothiol, or disulfide stress, OxyR becomes rapidly activated and induces the transcription of its target genes (3, 24, 63). Identified target genes encode for enzymes responsible for the degradation of peroxides (*katG*, *ahpC*, *ahpF*), for balancing the redox environment of the cell (*gor, grx1, trxC*) as well as for regulatory proteins (*fur*, *rpoS*, *flhA*) and the small noncoding, regulatory RNA *oxyS* (74).

The OxyR protein consists of an N-terminal DNA binding domain and a C-terminal oligomerization and regulatory domain. The C-terminal regulatory domain contains conserved cysteines that confer the redox sensitivity in OxyR (41, 42). Under normal reducing conditions, all six cysteines in OxyR are reduced and OxyR is inactive. Reduced OxyR is tetrameric and bound to the DNA, but is in a conformation that is unable to interact with RNA polymerase and to induce gene transcription (42). Upon exposure of OxyR to oxidative stress *in vivo* and *in vitro*, OxyR's cysteines are quickly modified. This leads to major conformational rearrangements and to the activation of the transcription factor. OxyR possesses two highly conserved cysteines (Cys199 and Cys208), of which only Cys199 appears to be absolutely essential for OxyR's

redox regulation and activation (42). A Cys199Ser OxyR mutant protein is locked in the reduced conformation, and cells are as hypersensitive to $H_2O_2$ as are cells that harbor an *oxyR* deletion. Optimal transcriptional activity, however, also requires the presence of Cys208. Mutant OxyR missing all four nonconserved cysteines (OxyR4C ⇒ A) has wild type activity *in vitro* and *in vivo* (73).

To elucidate the activation mechanism of OxyR, mass spectrometry and *in vivo* thiol-trapping techniques were applied (3, 73). These experiments, which were performed with the quadruple mutant protein OxyR4C ⇒ A, showed that the activation of OxyR is paralleled by the formation of an intramolecular disulfide bond connecting Cys199 and Cys208 (3, 73). The disulfide bond in OxyR is either formed directly by the action of $H_2O_2$ or by changes in the redox conditions of the cell (3). The cellular redox conditions have to become very oxidizing to activate OxyR because OxyR was found to have a rather high redox potential [−185 mV (73)]. This quite oxidizing redox potential can be achieved by mutating components of the thioredoxin and glutaredoxin pathway (3).

Based on observations that Cys199 plays a more critical role in the activation process of OxyR, it was proposed that oxidants like $H_2O_2$ react first with Cys199, resulting in the formation of a sulfenic acid intermediate. Cys199's sulfenic acid was then thought to undergo a nucleophilic attack on Cys208, yielding an intramolecular disulfide bond (73). This model makes Cys199 the primary $H_2O_2$ sensor. Structural analysis of OxyR's regulatory domain showed that the amino acids that flank Cys199 provide a positively charged environment, which is known to increase the reactivity of sulfur atoms, and could be the reason for OxyR's extremely high $H_2O_2$ sensitivity (7). Even within the reducing environment of the cytosol, 5 μM exogenously added $H_2O_2$ is sufficient to oxidize OxyR *in vivo* (3). The fact that reduction of OxyR is relatively slow compared with its oxidation also ensures the accumulation of oxidized and active OxyR in the overall reducing cytoplasmic milieu in response to low concentrations of $H_2O_2$. This slow rate of OxyR reduction is probably due to a stabilizing structural microenvironment that is formed upon formation of the disulfide bond (7). It allows OxyR's activity to peak at ∼10 min after $H_2O_2$ induction and then to only slowly decrease over the next 60 min in wild-type cells (73). The reduction and deregulation of OxyR has been shown to be conducted by members of the glutaredoxin system (3, 73).

Interestingly, oxidized, active OxyR has the same DNA binding affinity as reduced inactive OxyR. But whereas reduced OxyR binds only to two adjacent major DNA grooves, oxidized OxyR binds to four (65). Oxidation of OxyR appears to change the conformation in a way that exposes additional DNA binding residues. It results in a conformation that allows now the transcription factor to cooperatively interact with RNA polymerase (RNAp), an essential prerequisite to induce gene transcription. The major conformational changes that occur upon the oxidation and activation of OxyR became visible when the crystal structure of the oxidized and reduced forms of the regulatory OxyR domain were solved (7). In the oxidized form, the two cysteines form the expected disulfide bond. Upon reduction of OxyR, however, the two critical cysteines move 17 Å apart from each other in a process that causes a change in the overall protein fold and the oligomeric association (7).

That disulfide bond formation might not be the only way of activating OxyR became evident by recent results that showed that oxidative stress treatment of OxyR causes exclusive modification of Cys199 (34). The resulting Cys199 modification depends on the oxidant used and includes *S*-nitrosylation (SNO), *S*-hydroxylation (SOH), and *S*-glutathionylation (SSG). Interestingly, the individual OxyR species varied significantly in their conformation, DNA binding properties, and transcriptional activities *in vitro*, leading the authors to conclude that Cys199 plays an important role in "micromanaging" the activity of OxyR. OxyR-SNO, for instance, showed a cooperative binding to various promoters with very low transcriptional activity, whereas OxyR-SSG revealed a high-affinity, noncooperative binding with high transcriptional activity (34). This led the authors to the conclusion that OxyR might be able to differentially process different redox signals and produce distinct cellular responses. This very interesting concept, which awaits *in vivo* verification, would provide the cell with a highly sophisticated regulation mechanism (34).

## TURNING ON THE CHAPERONE Hsp33: BUILDING A HIGH-AFFINITY SUBSTRATE BINDING SITE

Hsp33 is a highly conserved heat shock protein that has been found in the cytosol of >50 procaryotic organisms. Recently, the first eucaryotic members have been identified in the mitochondria of *Chlamydomonas reinhardtii* and *Dictyostelium discoideum*. Hsp33 appears to be the first heat shock protein with dual regulation modes: on the transcriptional level, the Hsp33 gene (*hslO*) is under heat shock control (55), and on the posttranslational level, the Hsp33 protein is under oxidative stress control (30).

Functional studies revealed that Hsp33 is a very potent molecular chaperone when oxidized. In this conformation, Hsp33 has a very high affinity for protein folding intermediates and prevents their nonspecific aggregation. This protects cells against the otherwise lethal consequences of oxidative stress (30). Reduced Hsp33, on the other hand, does not exert any significant chaperone activity.

The redox switch in Hsp33 is composed of four highly conserved cysteines that are located in the C-terminal part of the protein. They are arranged in a Cys-X-Cys-X$_{27-32}$-Cys-X-X-Cys motif (30). In the reduced, inactive protein, the four conserved cysteines bind zinc with a very high affinity in a presumably tetrahedral coordination, thereby forming a novel zinc-binding motif (31). The zinc binding provides a very high stability and makes the domain a very stable folding unit. It is resistant to proteolytic digests, and even unfolding Hsp33 with 6 *M* guanidinium does not cause zinc release (31). Treatment with $H_2O_2$ or hydroxyl radicals, however, causes the formation of two intramolecular disulfide bonds and the immediate release of zinc (30). This occurs upon exposure of Hsp33 to $H_2O_2$ *in vitro* and upon oxidative stress treatment of *E. coli* cells *in vivo* (30). The two disulfide bonds that are formed in this process connect the next neighbor cys-

teines, Cys232 with Cys234 and Cys265 with Cys268 (4). The oxidized, monomeric Hsp33 is structurally stable and able to recoordinate zinc with the same high affinity upon return to reducing conditions.

Oxidation-induced disulfide bond formation is crucial, but is only the first step in Hsp33's activation. It is crucial because it leads to the structural rearrangements in Hsp33 that allow the chaperone to dimerize (Fig. 1). Only the dimeric Hsp33 reveals a very high affinity for protein folding intermediates (23). Disulfide bond formation, loss of the zinc, and dimerization cause significant changes in Hsp33's secondary and tertiary structure, as demonstrated by spectroscopic measurements and native gel electrophoresis (23, 54). It leads to the exposure of extensive hydrophobic surfaces in Hsp33 that are not exposed in its reduced state (54). This suggested that oxidation of Hsp33's cysteines and subsequent dimerization cause the formation of a hydrophobic substrate binding site that is capable of interacting with protein folding intermediates (67). Analysis of the crystal structure of dimeric Hsp33 revealed two putative substrate-binding sites (33, 67). One is a 10-stranded β-sheet saddle that is formed across both monomers and consists of a number of highly conserved, hydrophobic, and mostly uncharged residues. The second potential substrate binding site is a long groove that also lies across the two monomers and that is also composed of numerous hydrophobic niches and crevices that have the potential to interact with folding intermediates (33, 67). Whereas the activation process of Hsp33 is fairly well understood, the inactivation process of oxidized Hsp33 and the fate of substrate proteins that are so tightly associated with active Hsp33 still remain to be elucidated.

What role does zinc play in the redox regulation of Hsp33? Experiments with metal free Hsp33 revealed that zinc, a redox-inert metal, appears to take a rather active part in Hsp33's activation process. In the absence of bound metal, Hsp33's activation process was found to be slow and incomplete (31). Zinc may enhance the reactivity of the cysteines to which it is bound by stabilizing the negative charges on the thiolate anions, thereby lowering their p$K_a$ values and making them more reactive to $H_2O_2$ at near-neutral pH (25). Because the C-terminal thiol-rich zinc binding region of Hsp33 did not crystallize (33, 67), structural information about this region is not available. Sequence analysis, however, revealed that the zinc-coordinating and redox-active cysteine pairs of Hsp33 are surrounded by conserved positively charged amino acids, residues that are known to favor disulfide exchange reactions (**F**-**K**-$C_{232}$-T-$C_{234}$-S**R**- E-**R**-X$_{25}$-**H**-C-D-**Y**-C-G-N-**H**-**Y**, conserved residues shown in bold). Adjacent aromatic amino acids may also play a role by stabilizing disulfide bonds via the formation of S-π complexes (60, 68). These properties, in combination with the close proximity of the thiol groups in the tertiary structure due to zinc coordination and a potential accessibility of the zinc site to oxidants, may prime the cysteines for very rapid reactions with oxidants.

Over the past few years, it became evident that Hsp33 is only one member of a growing group of redox-regulated proteins that use thiol-rich zinc centers as their redox switch. These include a number of eucaryotic zinc finger transcrip-
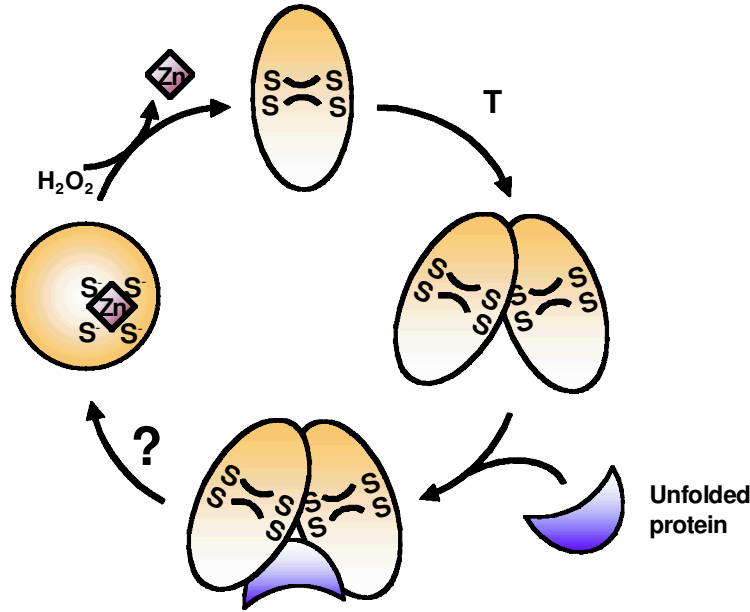
**FIG. 1.  Oxidative activation of the molecular chaperone Hsp33.** Under nonstress conditions, Hsp33 is predominantly monomeric, reduced, and inactive (circle). All four conserved cysteines coordinate a zinc atom in a novel zinc-binding motif. Oxidative stress causes the oxidation of Hsp33's cysteines. The protein releases zinc, and two intramolecular disulfide bonds form. This causes major structural rearrangements in Hsp33 (oval). In a highly temperature- and concentration-dependent process, Hsp33 dimerizes and forms its high-affinity substrate binding site. Active Hsp33 is now able to bind unfolding proteins and to efficiently prevent their nonspecific aggregation processes efficiently.

tion factors (*e.g.,* Sp1, Egr-1, GR) (68), protein kinase C (35), c-Raf kinase (27), and the zinc storage protein metallothionein (29). Metallothionein uses the oxidative switch to transfer zinc. Protein kinase C and c-Raf kinases are activated by thiol oxidation, whereas eucaryotic transcription factors become inactive when oxidized. This sensitivity of important signal transduction molecules toward oxidative modifications has major physiological implications for all those processes that are accompanied by changes in the redox state of the cytosol, such as aging and cancer (46, 70).
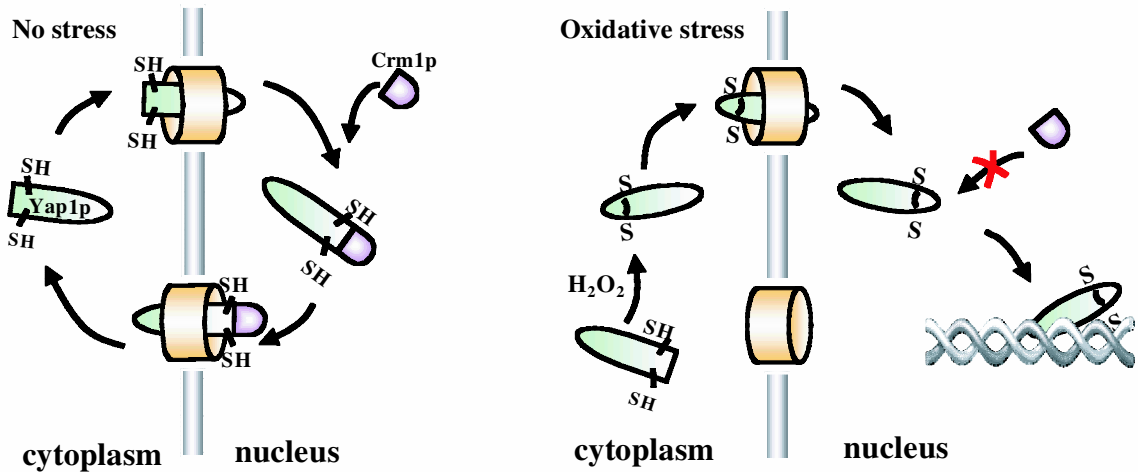


**FIG. 2.  Translocation cycle of Yap1p.** The redox-regulated transcription factor Yap1p is kept reduced by thioredoxin under normal growth conditions. Upon oxidative stress, one or more disulfide bonds form. Both the reduced and oxidized species migrate from the cytoplasm into the nucleus. The translocation back into the cytoplasm, however, is selective. Only reduced Yap1p can form a complex with the export receptor Crm1p and is transported back into the cytoplasm. The disulfide bond(s) in oxidized Yap1p abolish the ability of Yap1p to interact with Crm1p, and oxidized Yap1p accumulates in the nucleus. This results in the initiation of gene transcription.

## ACTIVATING RsrA: LETTING GO OF THE SIGMA FACTOR

RsrA (regulator of *sigR*) is the negative regulator of the antioxidant defense in the gram positive actinomycete *Streptomyces coelicolor* (32). In the reducing environment of the cytosol, the anti-sigma factor RsrA is active. It binds specifically to the sigma factor $\sigma^R$ (SigR) (est. $K_D = 1.1 \times 10^{-8}\ M$) and prevents SigR's interaction with its target gene promoters and, thus, the initiation of transcription (51). Upon exposure of RsrA to diamide-induced disulfide stress (36) or $H_2O_2$ induced oxidative stress, RsrA becomes rapidly inactivated. This results in a loss of affinity for SigR and to the release of the sigma factor (50). SigR can now bind to the DNA and induce the expression of a number of redox-balancing proteins such as thioredoxin and thioredoxin reductase, components of cysteine and molybdopterin biosynthesis, as well as the transfer messenger RNA ssrA and the ppGpp synthetase RelA (52). This restores the reducing character of the cytosol and effectively protects the organisms against disulfide stress. Similar systems have been identified in *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Rhodobacter spaeroides* (10, 43, 48).

RsrA is an 11.7-kDa protein with seven cysteines. Two of these cysteines form part of a highly conserved His-$X_3$-Cys$_{41}$-X-X-Cys$_{44}$ motif that is located near the N-terminus of the protein (32). The redox sensitivity of RsrA's functional activity and the presence of two highly conserved cysteines in a motif that is often found in proteins associated with thiol–disulfide exchange reactions (*e.g.,* Cys-X-X-Cys) suggested that the change in RsrA's activity is initiated by disulfide bond formation. Mass spectrometry experiments indicated that RsrA is indeed capable of forming three disulfide bonds upon oxidation *in vitro* (32). *In vivo*, however, only the three conserved cysteines (Cys11, Cys41, and Cys44) appear to be important. This became evident when single cysteine mutations were introduced into RsrA, showing that the individual mutation of either Cys11, Cys41, or Cys44 led to the complete inactivation of RsrA and to the constitutive overexpression of the antioxidant genes (51). This was somewhat surprising because wild-type RsrA is active in the reduced state, and it was assumed that preventing the formation of the potential disulfide bond(s) by mutating individual cysteines should render RsrA constitutively active rather than inactive. It suggested, however, that these cysteines are not only important for the redox sensitivity of RsrA, but also for the direct interaction with the SigR. It is, therefore, conceivable that the cysteines are located at or near the binding interface and essential for SigR binding. Disulfide bond formation and concomitant structural changes could block these residues or even bury them inside the protein and cause the release of the sigma factor (51). Another scenario could be that the observed zinc binding is involved in the interaction of reduced RsrA and SigR (51). Based on the arrangement of the conserved cysteines and histidines in RsrA, it seems likely that zinc is coordinated by at least some of the conserved thiolate anions (51). Mutation of the individual cysteines or oxidation-induced disulfide bond formation would abolish the zinc binding capacity of RsrA and could lead to the release of SigR.

The activation of SigR peaks within 10 min of exposure of the bacteria to disulfide stress and then slowly subsides until prestress levels of the antioxidant gene expression is reached (32). This is very similar to the activation pattern of OxyR. *In vitro* oxidized RsrA could regain its activity upon reduction by purified TrxA (32). As TrxA and TrxC (51) are under SigR control, this would provide an ideal mechanism of autoregulation for the RsrA–SigR system.

## OXIDIZING Yap1p: CHANGING HOME

In the budding yeast *Saccharomyces cerevisiae*, the response to oxidative stress (*e.g.,* $H_2O_2$) involves the transient overexpression of >115 proteins and the rapid repression of 52 proteins (21). At least 70 of these genes appear to be under the control of the transcription factor Yap1p, a 650-amino acid long bZIP DNA binding protein of the AP-1 family (19, 44, 57). Similar to OxyR in procaryotic organisms, Yap1p is responsible for the expression of genes encoding for catalase, thioredoxin, and thioredoxin reductase, enzymes that are essential for the cellular defense against oxidative and disulfide stress (44). Yeast strains lacking the *yap1* gene are significantly more sensitive toward $H_2O_2$ and diamide treatment and are unable to grow on media with elevated cadmium concentrations (26, 57).

The activation of Yap1p upon oxidation by $H_2O_2$ or diamide is mediated by highly conserved cysteines and involves the formation of intramolecular disulfide bonds (14). In contrast to OxyR, however, where the oxidation-induced conformational change leads to a change in DNA binding and RNA polymerase interaction, oxidation of Yap1p changes its subcellular localization (38, 40). Upon oxidation, Yap1p accumulates in the nucleus. This leads to the induction of gene transcription (Fig. 2) (for review, see 66).

The redox-active cysteines in Yap1p are clustered in two cysteine-rich domains (CRD) containing three cysteines each (11, 38). The n-CRD (Cys303, Cys310, Cys315) is located in the middle of the protein, whereas the c-CRD (Cys598, Cys620, Cys629) is close to the C-terminus of Yap1p. Embedded within the c-CRD is also a noncanonical leucine-rich nuclear export signal (NES) that is directly responsible for Yap1p's normal distribution and indirectly responsible for its functional regulation. In cells grown under nonstress conditions, Yap1p migrates unrestricted into and out of the nucleus. Upon migration into the nucleus, Yap1p interacts via the NES with the export receptor Crm1p that leads to the immediate export of Yap1p from the nucleus (38, 39). This results in a low nuclear concentration and, therefore, a low constitutive activity. Upon exposure of cells to oxidative stress, it was proposed that Yap1p might form intramolecular disulfide bonds. This could lead to conformational changes that mask the NES, prevent Crm1p binding, and, finally, cause the accumulation of the transcription factor in the nucleus (39). *In vivo* thiol-trapping experiments of Yap1p supported this hypothesis by demonstrating the appearance of disulfide-bonded Yap1p species, which paralleled Yap1p's activation and nuclear accumulation (14). Mutagenesis studies suggested an important role for at least four of the six cysteines in Yap1p. Delaunay and co-workers proposed that Cys303

and Cys598 form part of the redox center in Yap1p and suggested that a disulfide bond might form that connects the N-terminus with the C-terminus of the protein (14). Disulfide mapping of *in vitro* oxidized Yap1p using mass spectrometric analysis appeared to agree at least in part with this assumption. Within 1 min of oxidation *in vitro*, a disulfide bond was observed that connects Cys598 with Cys620 (40). This might be sufficient for the transient accumulation of Yap1p in the nucleus. The disulfide bond that was suggested by Delauney and co-workers to play a critical role (Cys303 and Cys598/Cys629) was found upon extended oxidation of Yap1p. This disulfide bond might be important for the prolonged accumulation of Yap1p in the nucleus (40, 66).

Interestingly, different cysteine residues in Yap1p appear to be modified, depending on the oxidant used. Yap1p C303A and C598A mutant proteins, for instance, are unable to respond to $H_2O_2$ treatment with the usual nuclear redistribution and transcriptional activity, but rapidly accumulate in the nucleus when treated with the thiol-specific oxidant diamide (14). Moreover, mass spectrometric analysis revealed a different disulfide pattern in diamide-treated Yap1p than in $H_2O_2$-treated Yap1p (40). This suggested that Yap1p is able to differentiate between $H_2O_2$ and diamide (14). That certain redox-sensitive proteins are able to process different redox signals in distinct ways is not so surprising, given how differently oxidants work on proteins and on the cellular redox status. What is very interesting, though, is the possibility that the same protein exerts different specific activities depending on the nature of the redox modifications. This graded and differentiated response that depends on the redox signal has been suggested for the procaryotic transcription factor OxyR (34) and might indeed apply also for Yap1p. It will therefore be very interesting to compare the Yap1p-dependent gene expression pattern in response to $H_2O_2$ and diamide treatment and analyze to what extent the genes overlap or vary.

Analysis of Yap1p's mechanism of deactivation was performed in strains that are defective in either one of the two major redox balancing systems. Whereas the time course of $H_2O_2$-induced gene expression was largely unchanged in strains defective in the glutaredoxin system, strains defective in the thioredoxin pathway accumulated oxidized Yap1p in the nucleus even in the absence of exogenous oxidative stress (14). Addition of $H_2O_2$ caused a prolonged nuclear localization and stress response. As thioredoxin and thioredoxin reductase are both genes that are under Yap1p control, these results suggest that this system is used as a negative feedback mechanism. Interestingly, deactivation of diamide-oxidized Yap1p was unchanged in thioredoxin-deficient mutants, suggesting that not only the activation of Yap1p is oxidant-specific, but also the deregulation mechanism (66).

## FOUR DOWN AND MANY MORE TO COME

The ability of organisms to sense ROS has been shown to be very crucial for their survival under stress and nonstress situations. It is therefore not surprising that pro- and eucaryotic organisms have developed mechanisms to protect the cell against the damage these reactive molecules can cause. At the same time, however, it appears that cells have also adopted systems that utilize such changing ROS levels and redox conditions as regulatory signals.

In this review, we present a few selected redox-regulated proteins that have in common the presence of at least two, usually very conserved cysteine residues, whose redox potential and reactivity allow the rapid formation of a covalent disulfide bond upon direct oxidation by ROS or changes in the cellular redox state. What makes these selected proteins different are their individual reactions that follow the structural rearrangements in the proteins, and which usually accompany the oxidative stress-induced disulfide bond formation. The oxidation of reduced inactive Hsp33, for instance, uncovers a hydrophobic binding site that has a very high affinity for protein folding intermediates (for review, see 22). This allows Hsp33 to specifically prevent aggregation processes of unfolding proteins under oxidative stress conditions. Oxidation of OxyR, on the other hand, causes the exposure of new DNA binding sites, which leads to a rearrangement of OxyR on the DNA and a conformation that can now bind RNA polymerase and induce gene transcription (72). The third example describes the anti-sigma factor RsrA, which, upon oxidation and disulfide bond formation, loses its affinity for its partner protein $\sigma^R$ (14, 51). This causes the release of SigR and induces the oxidative stress response in *Streptomyces coelicolor*. Finally, the yeast transcription factor Yap1p loses its affinity for the nuclear export factor Crm1p upon oxidation. This leads to the accumulation of oxidized Yap1p in the nucleus, which causes the induction of the oxidative stress response in yeast (66).

Redox reactions of proteins that involve thiol–disulfide exchange have been observed over many years in many laboratories, and numerous reports have described the use of reducing agents in buffers to protect the proteins from air oxidation. These redox reactions have never been interpreted as potentially very fast and elegant ways to regulate protein activity, but have usually been seen as purification artifacts (2). Given the lower oxidative power of air oxygen compared with the extreme reactivity of hydroxyl radicals, however, native proteins that are easily oxidized by air oxygen *in vitro* are probably even more easily oxidized by hydroxyl radicals *in vivo*. A general disbelief that specific disulfide bond formation could occur in cytosolic proteins and the lack of reagents that allowed direct monitoring of disulfide bond formation *in vivo* fueled this assumption and made this mode of functional regulation go unnoticed for many years (2). Only with the development of *in vivo* thiol-trapping reagents (71), mass spectrometric approaches, and suitable *in vivo* test systems (61) has the *in vivo* proof of oxidative stress-induced disulfide bond formation been possible. These experiments showed that oxidative stress-induced disulfide bond formation is indeed a very fast and reversible process that causes the quick inactivation of some proteins and the rapid activation of others.

Disulfide bond formation is, however, only one out of a variety of mechanisms that have been identified over the past few years to play a role in the redox regulation of protein function. The utilization of redox-active metal cofactors is another very commonly used strategy (*e.g.,* FNR, SoxR) (53, 58), as well as reversibly modifying single cysteines (*e.g.,*

Fos, Jun) (9). Presumably only a small percentage of these redoxregulated regulation mechanisms have been characterized so far, and further studies need to be performed to give us a more detailed understanding of these complex processes. This knowledge seems to be of increasing importance because oxidative stress has been shown to play a major role in the pathogenesis of diseases, and may even allow us to devise strategies to combat this down side of our aerobic life style.

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## NOTE ADDED IN PROOF

While this article was under review, Delaunay and coworkers provided evidence that hydroperoxide sensing of Yap1p is mediated by Gpx3p, a glutathionine peroxidase-like protein of yeast (15). Upon exposure of cells to $H_2O_2$, a transient intermolecular disulfide bond between Cys36 of Gpx3p and Cys598 of Yap1p is formed. This disulfide bond is then resolved to an intramolecular disulfide bond connecting Cys303 and Cys598 in Yap1p. The requirement for Gpx3p seems specific for $H_2O_2$ sensing of Yap1p because diamide sensing of Yap1p, for instance, is unaffected in cells lacking the *gpx3* gene.

## ABBREVIATIONS

CRD, cysteine-rich domain; GOR, glutathione oxidoreductase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; $H_2O_2$, hydrogen peroxide; NES, nuclear export signal; ROS, reactive oxygen species; RsrA, regulator of *sigR*; SigR, sigma factor $\sigma^R$; SNO, *S*-nitrosylation; SOH, *S*-hydroxylation; SSG, *S*-glutathionylation; Trx, thioredoxin.

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Address reprint requests to:
*Ursula Jakob*
*Molecular, Cellular and Developmental Biology*
*University of Michigan*
*830 North University*
*Ann Arbor, MI 48109-1048*

*E-mail:* ujakob@umich.edu

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